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Polymer materials for enzyme immobilization and their application in bioreactors

*Yan Fang, Xiao-Jun Huang, Peng-Cheng Chen & Zhi-Kang Xu**

MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China

Enzymatic catalysis has been pursued extensively in a wide range of important chemical processes for their unparalleled selectivity and mild reaction conditions. However, enzymes are usually costly and easy to inactivate in their free forms. Immobilization is the key to optimizing the in-service performance of an enzyme in industrial processes, particularly in the field of non-aqueous phase catalysis. Since the immobilization process for enzymes will inevitably result in some loss of activity, improving the activity retention of the immobilized enzyme is critical. To some extent, the performance of an immobilized enzyme is mainly governed by the supports used for immobilization, thus it is important to fully understand the properties of supporting materials and immobilization processes. In recent years, there has been growing concern in using polymeric materials as supports for their good mechanical and easily adjustable properties. Furthermore, a great many work has been done in order to improve the activity retention and stabilities of immobilized enzymes. Some introduce a spacer arm onto the support surface to improve the enzyme mobility. The support surface is also modified towards biocompatibility to reduce non-biospecific interactions between the enzyme and support. Besides, natural materials can be used directly as supporting materials owning to their inert and biocompatible properties. This review is focused on recent advances in using polymeric materials as hosts for lipase immobilization by two different methods, surface attachment and encapsulation. Polymeric materials of different forms, such as particles, membranes and nanofibers, are discussed in detail. The prospective applications of immobilized enzymes, especially the enzyme-immobilized membrane bioreactors (EMBR) are also discussed. [BMB reports 2011; 44(2): 87-95]

*Corresponding author. Tel & Fax: +86-571-8795-2605; E-mail: xuzk@zju.edu.cn
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INTRODUCTION

Enzymes are highly specific, efficient, and mild catalysts in fine-chemical or pharmaceutical synthesis, food processing and bioremediation. However, free enzymes, after being optimized via natural evolution and always catalyzing in complex metabolic pathways, are seldom suitable to be used in industrial catalysis where operational conditions are far from those in the natural biological environment (1, 2). In practical applications, enzymes are always attached or incorporated onto or into an inert, insoluble material (3). This protocol is called enzyme immobilization, which can overcome the instability and non-reusability limitations connected with the free enzymes, making continuous operations and simplified product purification feasible (4, 5).

However, during the immobilization process, the multipoint attachment to support unavoidably hampers the free conformation of enzymes and sometimes non-biospecific interactions of enzyme - support result the denaturation of enzyme protein, making the activity retention of the immobilized enzymes lower than 100% (6). It is thus important that the properties of supporting materials and immobilization processes should be well understood in order to improve activity retention of the immobilized enzyme. Based on their chemical compositions, supports can be divided into two categories: inorganic and polymeric materials. In recent years, there has been growing concern about the later for its good mechanical properties and being easily tailored according to specific requirements (7). Moreover, by moderating the surface chemistry of supports, we can improve the specific activity and stabilities of immobilized enzymes. A flexible spacer can be introduced onto the supports to offer the immobilized enzyme high freedom of movement as well as minimize unfavorable steric hindrance posed by solid supports. Modifying the surface chemistry towards biocompatibility is also commonly used for promoting the activity of immobilized enzymes. By mimicking the biological environment in which enzymes flourish in nature, including using natural polymers such as cellulose and collagen directly as supports, the immobilized enzymes are supposed to be able to stabilize their structure and retain their activities.

When it comes to the applications of immobilized enzymes,

much attention has been paid to bioreactors. Among different types of bioreactors, enzyme-immobilized membrane bioreactors (EMBR) stand out as a specific mode for running continuous processes (8, 9). In these processes, enzymes are separated from end products using a selective membrane, creating a simultaneously procedure of enzymatic catalysis and product separation. Depending on the case that enzyme molecules be immobilized onto the membrane surface or inside its porous structure, different kinds of EMBR may be designed. Up to now, EMBR have been widely studied in the field of fine-chemicals synthesis, food industries or even wastewater treatments.

Among the great variety of enzymes, lipases have gained much attention for being capable of catalyzing a wide range of popular reactions such as alcoholysis, hydrolysis, trans-esterifications and enantiomer resolution (10). For practical applications, like other enzymes, lipases often go through immobilization process in order to elongate their lifetime and improve their stabilities. Besides, versatility of the lipase-immobilized bioreactors make them quite unique in drug synthesis, food making, detergent and cosmetics industries. In this paper, lipase is taken as the model enzyme to present the recent advances in using polymer materials (pristine and modified) of different forms (particles, membranes and nanofibers) as supports for enzyme immobilization. The immobilization methods like surface attachment and encapsulation, including some modification strategies like spacer arm introduction and biomimicking, are discussed in detail. Furthermore, application of the enzyme-immobilized bioreactors, especially EMBR is also discussed.

Surface Attachment Immobilization of Enzymes on Polymer Supports

Enzyme immobilization on particles

In recent years, considerable efforts have been put into the studies of using polymer particles for enzyme immobilization. As the promising property of lipase activation at interfaces in the presence of hydrophobic interface, some tuned hydrophobic supports like polypropylene powder obtained with metallocenic catalysts (PPmet) were used for adsorption of *Candida rugosa* lipase (CRL) and it was found that ethanol pretreatment of the PPmet not only enhanced PPmet's affinity towards lipase aqueous solution but led to increased solid recovery (11). Following the same method, PPmet and commercial polypropylene particles obtained with Ziegler-Natta catalysts (PP_{ZN}) in the diameter range of 590-1,180 μm were also used for *Candida antarctica B* lipase (CALB) immobilization. And it was found that the adsorbed enzyme on PPmet showed relatively high activity and stabilities due to their porous surface and smaller particle size (12). In Chen's study, both macroporous poly (methyl methacrylate) resins and macroporous polystyrene resins with varied particle sizes (35 to 560-710 μm) were employed for CALB adsorption (13, 14). Compared with polymethacrylate resins having similar physical parameters, polystyrenic resins had higher affinity for CALB adsorption and provided CALB with a favorable microenvironment to orient and take on conformations that favors the active site's accessibility to substrates. Some pristine copolymer particles were also widely used as supports. Meanwhile, some acrylic resins can be directly used as supports for lipase immobilization due to their high porosity and high density of functional oxirane groups on the surface, as in the examples of Eupergit® C, which is a macroporous copolymer of N,N'-methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide (15, 16). Öztürk *et al*. prepared hydrophobic poly (2-hydroxy-ethyl methacrylate-*co*-N-methacryloyl-(l)-phenylalanine-methyl ester) (poly(HEMA- MAPA)) nanospheres by surfactant free emulsion polymerization of 2-hydroxyethyl methacrylate (HEMA) and N-methacryloyl-(l)-phenylalaninemethyl ester (MAPA) for adsorption of lipase in batch system, achieving phenomenally high enzyme loading (329 mg lipase/g nanosphere) and storage stability (17). Following the same polymerization method, poly(2-hydroxyethyl methacrylate-*co*-N-methacryloyl-(L)-tryptophane methyl ester) (poly(HEMA-MATrp)) nanospheres utilizing N-methacryloyl-(L)-tryptophane methyl ester (MATrp) as the hydrophobic monomer were also used for lipase immobilization (18). It was observed that enzyme could be reversibly adsorbed and desorbed without significant loss in adsorption amount or enzyme activity.

However, during the immobilization process, it is inevitably that multipoint attachment to the supports restrict the free movement of enzymes, and thus resulting into the activity and stability losses. In order to overcome this limitation, a flexible spacer is often introduced onto the supports. It has been mentioned that Eupergit® C resins can be directly used for lipase immobilization via their oxirane groups (15-19). Based on those functional groups, Foresti *et al*. pre-introduced 1,2-diaminoethane and glutaraldehyde as a spacer arm onto the surface of Eupergit® C following the immobilization of CRL. Compared to the direct lipase binding to Eupergit® C method, the latter increased the flexibility and activity of the immobilized enzymes (19). Similarly, in the study by Miletic *et al*., crosslinked macroporous hydrophilic poly (glycidyl methacrylate-co-ethylene glycol dimethacrylate) (poly(GMA-co-EGDMA)) were synthesized via suspension polymerization and then went through glutaraldehyde and cyanuric chloride modification process. It was shown that as the amount of glutaraldehyde or cyanuric chloride increased, the activity of immobilized CALB primary increased at first and then decreased (20, 21).

Surface modification towards biocompatibility is also commonly used for promoting the activity of immobilized enzymes. By mimicking the biological microenvironment where enzymes naturally flourished, the biomimetic systems are supposed to be able to stabilize the structure of enzymes and retain their activities. For example, amino acid modified chitosan beads (CBs) for immobilization of CRL were prepared by

activation of a chitosan backbone with epichlorohydrin followed by amino acid coupling. The immobilized lipase on the amino acid modified CBs showed activities higher than that of the unmodified CBs (22). In addition to grafting, blending is another effective method for biomimetic modification of supports. Blends of natural polysaccharide sodium alginate with gelatin provided beads with excellent biocompatibility for lipases immobilization (23). The immobilized enzymes showed high stability and reusability both in aqueous and reverse micellar media.

Further more, some nature polymers, with their excellent inert and biocompatible properties, are directly used as supports for enzyme immobilization. For example, sporopollenin, a highly stable natural polymer obtained from Lycopodium clavatum, was applied in adsorption of CRL. It was found that the stabilities of the immobilized lipase toward pH, temperature, storage and kinetic properties were enhanced significantly (24).

Enzyme immobilization on membranes

As an important unit operation, membrane separation exhibit unique advantages of high selectivity, high surface-area-per-unit-volume, and their potential for controlling the level of contact and/or mixing between two phases. Hence polymeric membranes (such as microfiltration and ultrafiltration membrane) for enzyme immobilization have been widely investigated.

Bayramoğlu *et al*. reported that poly(HEMA-MAPA) membrane was prepared by UV-initiated photopolymerization of HEMA and MAPA in a round glass mould with α-α′-azobisisobutyronitrile (AIBN) as an initiator and used for CRL immobilization by covalent bonding through carbodiimide activation (25). The amount of enzyme loading increased as the MAPA ratio increased in the membrane structure. And the activity of immobilized enzyme was found to be quite stable in repeated experiments. Similarly, Ye *et al*. prepared ultrafiltration hollow fiber membranes from poly(acrylonitrile-co-maleic acid) (PANCMA) for CRL immobilization with 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride/N-hydroxyl succinimide (EDC/NHS) as coupling agent (26). The activity of the immobilized lipases in the organic medium (heptane) was improved significantly. In addition, *Candida Cylindracea* lipase could be directly immobilized via glutaraldehyde crosslinking on the polysulfone and polyethersulfone asymmetric membranes (27).

Surface modifications have also been carried out on polymeric membrane to mediate the surface properties for enzyme immobilization. PPM is more interesting due to its hydrophobicity, well-controlled porosity, and chemical inertness as well as high potentials for comprehensive applications. To create an active and biocompatible interface for lipase immobilization, Gupta *et al*. modified PPM surface by radiation induced graft polymerization of GMA (28). The resulting epoxy was converted into a diethyl amino group as an anion-exchange medium to bind the lipase molecules. It was shown that a high operational stability was obtained for the immobilized lipase. Deng *et al*. also modified microfiltration PPM by tethering poly(γ-ethyl-L-glutamate) (PELG), poly(γ-stearyl-L-glutamate) (PSLG) and poly(α-allyl glucoside) (PAG) onto the surface for CRL immobilization (29, 30). It was found that the thermal stability of the immobilized enzyme was obviously improved on the modified PPM. Surface modification of chitosan membranes was also performed using the amidating reaction of amino groups on the membrane surface with various monomers activated by EDC/NHS (31). The experimental results showed the stability of immobilized lipase increased when compared to that of the free lipase.

In order to create a biocompatible interface for lipase immobilization, efforts have been made toward the biomimicking methodology. For example, Deng *et al*. tethered phospholipid analogous polymers (PAPs), containing hydrophobic octyloxy, dodecyloxy, and octadecyloxy groups (abbreviated as 8-PAP, 12-PAP, and 18-PAP, respectively) onto the surface of hollow fiber PPM to create a natural microenvironment for CRL immobilization (32). The activity retention of immobilized lipase increased from 57.5% to 74.1%, 77.5%, and 83.2% respectively for the 8-PAP-, 12-PAP-, and 18-PAP-modified membranes. Besides, the previous mentioned PANCMA membranes were biomemetic modified by natural macromolecule, chitosan, which was tethered on the surface of PANCMA membrane to prepare a dual-layer biomimetic support for CRL adsorption (33). The activity retention of the immobilized lipase on the chitosan-tethered membrane was higher than that by chemical bonding. Composite membrane prepared by coating the cellulose acetate (CA) layer on the hydrophobic polytetrafluoroethylene (PTFE) layer was also used for lipase adsorption by filtration process (34). The composite membrane was easily regenerated and lipases immobilized in the regenerated membrane remained a high activity and retained 80% residual activity after ten hydrolysis cycles.

Many natural polymers like regenerated cellulose and chitosan, which possess excellent membrane forming property, good thermal, mechanical stability and biocompatibility, have been successfully applied as membrane materials and therefore widely used for enzyme immobilization (35-37). The stabilities of the immobilized lipases were obviously improved on these natural polymeric membranes. In recent studies, egg shell membranes, which are a novel, robust, microporous, cost effective, easily available organic support material, were also utilized for lipase immobilization (38). It was found that the immobilized enzymes were stable even after 180 days of storage.

Enzyme immobilization on nanofibers

To achieve high enzyme loading and catalytic efficiency of enzymes for large-scale operation and application, polymer nanofibers have attracted continuous attention for their unique properties (39, 40). Compared with other nanostructured supports (e.g. mesoporous materials, nanoparticles), nanofibers have large specific surface which can provide relatively high

quantity of enzyme loading and high porosity giving the accessibility of active sites and low diffusion resistance necessary for high reaction rate and conversion (41, 42).

Lipases can be chemical covalently immobilized onto the nanofibers with functional groups on the surface. For example, nanofibrous membranes containing reactive carboxyl groups were fabricated from PANCMA by electrospinning (43). Then, CRL was covalently attached onto the nanofibrous membrane surface via the activation of carboxyl groups in the presence of EDC/NHS. It was found that, compared with the PANCMA hollow fiber membrane, the enzyme loading and the activity retention of the immobilized lipase on the nanofibrous membrane increased from 2.36 ± 0.06 to 21.2 ± 0.7 mg/g and from 33.9% to 37.6%, respectively. In the study by Huang *et al*., poly(acrylonitrile-co-2-hydroxy-ethyl methacrylate) (PANCHEMA) was also electrospun into nanofibrous membrane for lipase immobilization. The hydroxyl groups on the surface were activated with three chemicals: epichlorohydrin, cyanuric chloride and p-benzoquinone (44). It was shown that the observed enzyme loading and the stabilities of the immobilized lipase were obviously improved. PAN nanofibers were also used as supports for CRL immobilization by amidination reaction (45). The protein loading efficiency was quantitative and the activity retention of the immobilized lipase was 81% that of free enzyme. In order to further improve the enzyme loading and activity of the immobilized lipases, enzyme molecules and aggregates from solution could be cross-linked to the surface of PAN nanofibers by glutaraldehyde (46, 47). The immobilized lipases retained high stability, easy recoverability and reusability.

The flexibility and retention activity of immobilized enzyme can also be improved by introducing spacer arms onto the surface of nanofibers (48, 49). Wang *et al*. introduced PEG as the spacer arm onto the alkali-hydrolyzed cellulose nanofibers for covalent immobilization of lipase (50). The highly hydrophilic PEG layer ensured the conformational flexibility of immobilized enzyme by offering essential water. And thus the immobilized lipase presented high stability in organic media.

What is more, to increase the activity and stability of the immobilized enzymes, much attention has also been paid to surface modification of nanofibers towards biocompatibility. It has been mentioned earlier that the nanofibers electrospun from PANCMA was used for lipase immobilization (43). With availability of functional groups on the surface of the nanofibers, they can react with biomacromolecules such as chitosan or gelatin to build dual-layer biomimetic surface for lipase immobilization (51). It was shown that there is an increase of the activity retention of the immobilized lipase on both the chitosan-modified and the gelatin-modified nanofibrous membranes, compared to that on the nascent ones. Similarly, collagen or protein hydrolysate from egg skin (ES) was also tethered on the prepared poly(acrylonitrile-co-acrylic acid) (PANCAA) nanofibrous membranes for lipase immobilization (52). The stabilities of the immobilized lipases were obviously improved compared with the free one.

Phospholipids, as the principal components of natural biomembranes, have been proved to be inherently biocompatible with various proteins. Based on the biomimetics methodology, Huang *et al*. prepared novel nanofibers from the copolymer of acrylonitrile and 2-methacryloyloxyethyl phosphorylcholine (MPC) for lipase immobilization (53). It was found that the introduction of phospholipid moieties obviously enhanced the activity of lipase, and retained the enzyme loading through electrostatic interaction between phospholipid moieties and enzyme molecules. Generally, phospholipids anchored on the support surface provided stabilization and activation effects to the immobilized lipase. In addition to biomemetic modification of nanofibers by introducing natural biomacromolecules to the surface, the synthetic macromolecules such as PVP and PEG were used as additives to render the nanofibrous membranes biocompatibility favored by immobilized enzyme (54). The results showed that the activity of immobilized enzymes largely depends on the content of PVP or PEG in the nanofibers.

Natural polymers can be easily electrospun into nanofibrous

Table 1. Different structure forms of polymer supports for lipases immobilization

Structure forms	Support	Immobilization method	Protein loading (mg/g upports)	Activity retention (%)	Ref.
Particles	PMMA resins	Physical adsorption	100	43.0	(13)
Psrticles	poly(HEMA–MAPA) nanospheres	Physical adsorption	329	66.0	(18)
Particles	Eupergit® C resins	Chemical attachment	37.0	31.1	(15)
Particles	Chitosan beads	Physical adsorption	231.9 ± 14.3	10.4 ± 0.5	(22)
Membranes	PP membranes	Physical adsorption	82.7 ± 2.6	57.5 ± 2.8	(29)
Membranes	Poly(HEMA-MAPA) membranes	Chemical attachment	4.23	/	(25)
Membranes	PANCMA membranes	Chemical attachment	23.3	114	(26)
Membranes	Chitosan membranes	Physical adsorption	30	/	(37)
Nanofibers	PANCMA nanofibers	Chemical attachment	21.2 ± 0.7	37.6	(43)
Nanofibers	PANCHEMA nanofibrous	Chemical attachment	16.2 ± 1.1	40.6	(44)
Nanofibers	PANCMPC nanofibers	Physical adsorption	22.9 ± 1.5	76.8 ± 0.6	(53)
Nanofibers	Chitosan nanofibers	Chemical attachment	63.6	49.8	(57)

membranes and also be widely used as supports for enzyme immobilization (55-57). As reported by Lu and Hsieh, Cellulose (Cell) nanofibrous membranes prepared by nucleophilic reaction of the cellulose hydroxyl with the triazinyl chloride of Cibacron Blue F3GA (CB) ligand were used as affinity membranes for lipase immobilization (56). The CB-Cell bound lipase had similar catalytic rate and retained 86.2% activity as in its free form.

Table 1 summarizes the lipase immobilization results using polymeric materials in forms of particles, membranes and nanofibers as supports. In order to improve the activities and the stabilities of immobilized enzymes, different surface modification methodologies were adopted towards promoting the flexibility of immobilized enzymes and offering the enzymes a microenvironment analogous to that of the natural one. What's more, some natural polymers were also used as supports directly.

Encapsulation Immobilization of Enzymes in Polymer Supports

Encapsulation is another primary method for lipases immobilization. Yang *et al*. used organosilicone materials such as silica sol-gels prepared by polymerization of various silanizing agents including vinyl-trimethoxy silane, octyl-trimethoxy silane, γ-(methacryloxypropyl)-trimethoxy silane (MAPTMS) and tetraethoxysilane (TEOS) as supports for lipases entrapment (58, 59). It was found that the prepared enzyme by copolymerization of MAPTMS and TEOS exhibited higher activity. Further, additives were found to have a stabilizing effect on sol-gel entrapped enzymes. Yilmaz and coworkers encapsulated lipase in a sol-gel support prepared by polycondensation of TEOS and octyltrietoxysilane (OTES) with or without calix(n)arene, calix(n)-NH_2, calix(n)-COOH(n = 4, 6, 8) and sporopollenin compounds as additives (60, 61). The results indicated that the particularly calix(4,6)-NH_2, calix(6)-COOH and sporopollenin based encapsulated lipases had higher conversion and enantioselectivity compared to the sol-gel free lipase.

On the other hand, in order to improve the activity and stability of the encapsulated enzymes, encapsulations on natural polymers based hydrogel beads have gained much attention due to their excellnet biocompatibility. So far, polysaccharide hydrogel beads such as agarose beads, alginate beads, chitosan beads, κ-carrageenan beads, and nanogels of cholesterol-bearing pullulan (CHP) have been used as supports for lipases entrapment immobilization (62-65). Those entrapped lipases retained a high degree of activity in multiple reactions.

In recent years, much attention has also been paid to lipases entrapped in membranes or nanofibers. In Monier's study, CRL was entrapped in the modified photo-crosslinkable chitosan membranes and the immobilized lipase exhibited better thermal stability than the free one (66). Sakai *et al*. electrospun polystyrene nanofibers from a suspension of crude lipase powder in an N,N-dimethylformamide (DMF) solution of polystyrene (67). The encapsulated and moistened lipase showed 77% of residual activity after 10 cycles of use.

Potential Applications in Bioreactors

When it comes to the applications of immobilized enzymes, bioreactor is one of the hottest research points. Compared with fluidized-bed reactors, enzyme-immobilized membrane bioreactors (EMBR) stand out as a specific mode for combining product separation process with enzymatic catalysis in continuous processes. The selective membrane aims to separate the enzymes from the reaction products. Up to now, EMBR have been widely used in the field of fine chemicals synthesis (such as aminoacids, antibiotics, anti-inflammatories, anticancer drugs, vitamins, and optically pure enantiomer, etc.), food industries or even wastewater treatments (68-73).

As for lipase-immobilized membrane bioreactors, they are usually used in the field of non-aqueous phase catalysis such as synthesis (74-79), hydrolysis (80-82) and kinetic resolution of compounds (83-89). For example, Tan *et al*. fabricated a lipase-immobilized membrane bioreactor for synthesis of 2-ethylhexyl palmitate, obtaining an average esterification degree of 95% and a product purity of 98% (77). Besides, it was mentioned by Huang *et al*., the PANCHEMA electrospun nanofibrous membranes could be used for the covalent immobilization of CRL (45). Further continuous hydrolysis of p-nitrophenyl palmitate (p-NPP) was carried out with the enzyme-immobilized nanofibrous membrane bioreactor and a steady hydrolysis conversion was obtained under optimum service conditions (see schematically in Fig. 1). Another EMBR for enantioseparation of (R,S)-ketoprofen via CALB as biocatalyst were investigated by Aboul-Enein *et al*. (88). It was shown that im-

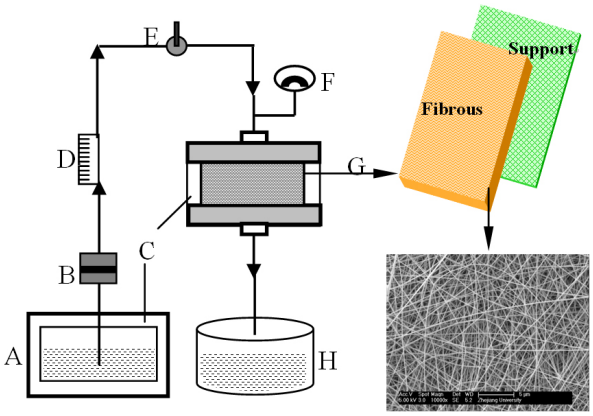

Fig. 1. Immobilized CRL based PANCHEMA electrospun fibrous membranes bioreactor for hydrolysis of p-NPP. (A) substrate reservoir; (B) pump; (C) temperature controller; (D) flowmeter; (E) valve; (F) pressure gauge; (G) lipase-immobilized fibrous membrane; (H) product.

mobilization of CALB in the EMBR was able to reduce the amount of enzyme required for the enantioseparation of (R,S)-ketoprofen. In addition, the EMBR assured higher reaction capacity, better thermal stability, and reusability.

However, it should be noted that although EMBR have advantages in the productivity, quality and diversity of productions, the applications at industrial scale are rare due to decay of enzyme activity over time, which is caused by the various reasons of steric hindrance effects (the active site of the enzyme molecule may be distorted by the immobilization process) and interfacial limitation phenomena (the diffusion of substrates or products in the vicinity of the surface on which the catalyst molecules are immobilized may be very low). In order to solve these problems, both new kinds of polymer membranes and novel enzyme immobilization methods should be adopted.

CONCLUSIONS

Polymer materials have been proven to be important supports for lipases immobilization. With the development of polymer synthesis, more and more novel polymers with excellent properties are prepared for lipase immobilization. The EMBR technology is one of the most important applications of those immobilized enzymes and it has been widely used in the field of synthesis, hydrolysis and kinetic resolution of compounds. However, most of the EMBR are only tools from laboratory rather than systems with broad industrial applications. Future in view, on the basis of new polymer materials and novel methods for enzyme immobilization, the performance of the immobilized enzymes may be improved dramatically and the applications of EMBR at industrial scale are also promising.

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